A HEMOLYTIC SEROLOGIC REACTION WITHOUT COMPLEMENT AS A POSSIBLE TEST FOR SYPHILIS*

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The following investigation is based on the phenomenon first observed by Gengou (2) that sheep cells would be agglutinated and hemolyzed when barium sulphate, a chemically inert substance, was introduced into a physiological saline solution; but, in the presence of a trace of serum this phenomenon was inhibited.

An extract of old, crude, partially oxidized egg lecithin, was substituted for the barium sulphate of Gengou's experiment because lecithin, as is known, has the property of combining with the reagin in syphilitic serums (3). And, it has also been known for some time that phospholipids (lecithin and cephalin) have a hemolytic effect on erythrocytes (1).

The purpose of this experiment was to determine if a hemolytic test for syphilis could be developed in the absence of amboceptor and complement employing an "antigen" with hemolytic properties.

The preliminary experiments with an ether-soluble extract of crude egg lecithin showed that sero-negative serums permitted the hemolysis of the sheep cells by the lecithin while sero-positive serums, in the same dilution, inhibited hemolysis of the sheep cells. (Refer to Charts A and B.) In order to increase the sensitivity of the reaction cardiolipin and cholesterol were added to the above mentioned extract.

EXPERIMENTS

To determine the effect upon the hemolytic activity of the crude lecithin extract with (1) an increase in colloidal dispersion, (2) an increase in the concentration of the colloidal suspension and (3) changes in the volume of sheep cell suspension employed the following experiments were performed.

Experiment I

Alcoholic solutions of lecithin were prepared in concentrations of 1.5%, 3%, 4.5%, and 6%. A colloidal suspension in 0.85% sodium chloride solution was prepared from each alcoholic solution so that the weight of lecithin in each suspension was identical; the variation being in the difference of colloidal dispersion. Into each of four separate test tubes 0.4 ml. of each colloidal suspension was pipetted. To each was then added 1.6 ml. of 0.85% saline. The tubes were

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then placed in an incubator at 37° C for thirty minutes. 1.0 ml. of a 5% suspension of sheep cells was then pipetted into each tube; tubes shaken and replaced in the incubator. The tubes were then observed at frequent intervals for hemolysis; the time required for complete hemolysis to occur in each tube was recorded (Graph 1). In it will be noted that hemolysis occurs first in the tube containing the colloidal suspension of lecithin prepared from the 6% alcoholic solution; the length of time required for hemolysis to take place increasing progressively with the decrease in the concentration of the original alcoholic solution.

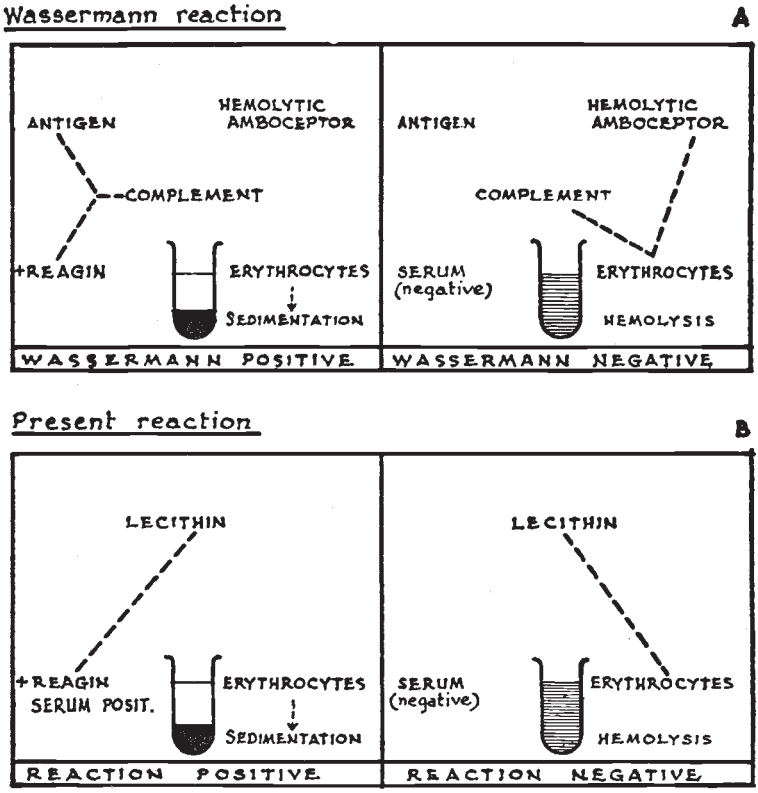


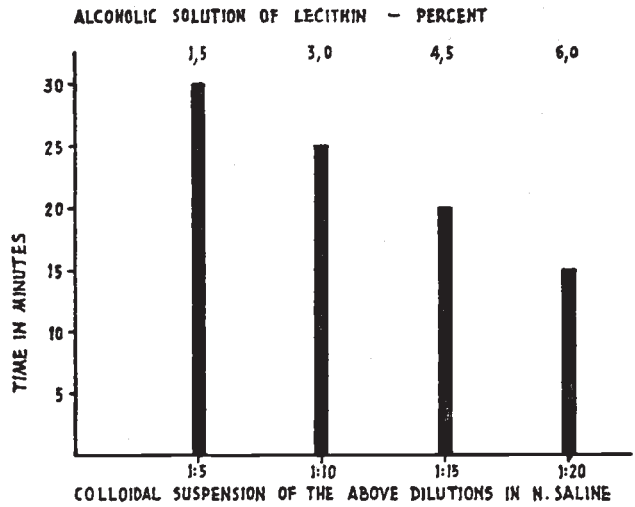
CHART A. and Chart B. Diagrammatic comparison of the mechanisms of the Wassermann and present reaction

Experiment II

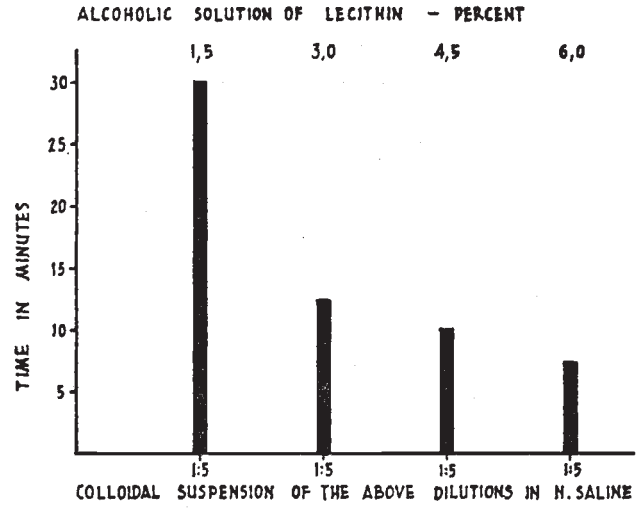
A 1:5 colloidal suspension in 0.85% saline was prepared from each of the alcoholic solutions of lecithin mentioned above. Each tube therefore contains increasing concentrations of lecithin. When the identical procedure as described in Experiment I was followed, it was observed that hemolysis occurred first in that tube which contained the greatest concentration of lecithin; the time required for hemolysis to take place increasing progressively with a decrease in the concentration of lecithin (Graph 2).

Experiment III

From the 3% alcoholic solution of lecithin a 1:40 colloidal suspension in 0.85% saline was prepared. To each of three test tubes 0.4 ml. of this suspension was



GRAPH 1. Showing the increase of the hemolytic power with the increase of the colloidal dispersion

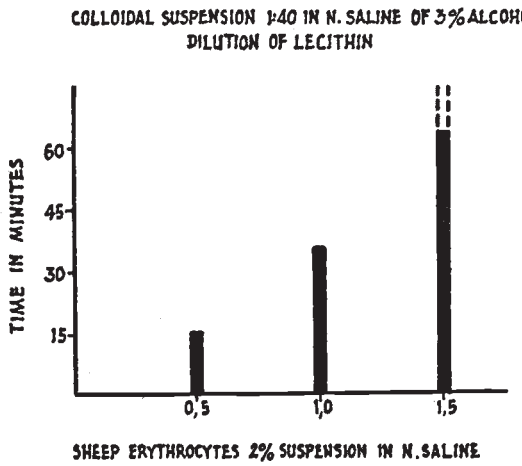


GRAPH 2. Showing the increase of the hemolytic power of Lecithin with the increase of Lecithin concentration

pipetted. Then 2.1 ml., 1.6 ml. and 1.1 ml., respectively, of 0.85% saline was added to these tubes. After incubation at 37° C for 30 minutes, 0.5 ml. of a 5% sheep cell suspension was added to the first tube, 1.0 ml. to the second and 1.5

ml. to the third (total volume 3 ml. in each tube). Tubes were shaken and returned to the incubator. Time required for hemolysis to occur was noted. From Graph 3 it will be seen that hemolysis occurred in the first tube after 15 minutes, in the second after 35 minutes and that in the third there was no hemolysis even after one hour. It may therefore be deduced that the hemolytic activity of lecithin varies in inverse proportion to the volume of sheep cell suspension employed.

To summarize the results of the above experiments, it may be postulated that the hemolytic activity (HA) of the lecithin is directly proportionate to the lecithin concentration (c), to its degree of colloidal dispersion (d), and to the time (T), and in inverse proportion to the volume of sheep cell suspension (v); the



GRAPH 3. Showing the decrease of hemolytic power of Lecithin with the increase of the volume of Erythrocytes

factors of temperature (37° C) and total volume of reagents (3 ml.) being constant. This ratio may be expressed with the following formula:

$$HA = \frac{c \cdot d \cdot T}{v}$$

or, more simply, when the time (30 min.) and the volume of sheep cell suspension (1 ml.) are constant as:

$$HA = c \cdot d$$

A *hemolytic unit* may be defined as that amount of lecithin, in any particular colloidal state, which is just capable of hemolyzing 0.5 ml. of a given erythrocyte suspension under standard conditions of temperature and time. In the performance of the actual test *two hemolytic units* are employed; that is, the concentration of lecithin and its colloidal state is selected on its ability to hemolyze 1 ml. of the sheep cell suspension although 0.5 ml. of the sheep cell suspension is used in the test proper.

TECHNIC OF THE TEST PROCEDURE

Preparation of Serum

The serum is obtained in the usual manner by centrifugation of a clotted blood sample. The serum is inactivated at 56° C. in a water bath for 30 minutes.

Preparation of Antigen

1. Add 6 grams of commercial lecithin to 200 ml. of anesthesia ether, stirring until dissolved.
2. Pour this solution into a separatory funnel. Add 25 ml. distilled water. Shake.
3. Allow two layers to form. The upper will be light amber and the lower dark brown.
4. Let stand at room temperature for 36–48 hours with occasional shaking.
5. Discard the lower (dark brown) water-soluble fraction.
6. Pour the ether-soluble fraction into a previously weighed Erlenmeyer flask and place in a waterbath at 60° C. until the ether has evaporated.
7. Transfer the flask to a water bath at 90°–95° C. for 15–20 minutes to evaporate any residual ether.
8. Remove the flask from the water bath and allow to stand at room temperature until the lecithin has hardened.
9. With an alcohlo-moistened swab remove any drops of water that may be adhering to the sides of the flask as it must be absolutely dry.
10. Weigh the flask and compute the weight of lecithin contained therein.
11. Prepare a 3% solution of the lecithin in 96% alcohol. Add cholesterol to a concentration of 0.9% (Difco, ash-free pure cholesterol).
12. Allow to stand at room temperature for 1–2 days in a dark bottle.
13. Filter through fat-free filter paper. This filtrate is the "antigen".

Preparation of the Colloidal Suspension

Measure 21 ml. of a NaCl solution (Eagle Saline Solution) pH 7.4 in a 100 ml. graduated cylinder (diameter approximately 2.8 cm.).

Add 0.175 ml. of a 1:10 dilution of Rein-Bossak Cardiolipin Antigen (diluted with 96% alcohol) to 1.0 ml. of "antigen". Mix and measure out 1.0 ml. of this "antigen" mixture in a 1 ml. pipette.

Allow the first 0.1 ml. to very slowly run down the side of the cylinder containing the Eagle saline solution. Invert the cylinder once. Then let remainder slowly flow down the side of the cylinder. Place the palm of the hand over the opening of the cylinder. Slowly tilt the cylinder to a horizontal position allowing the colloidal suspension to undulate back and forth very slowly several times. Slowly bring the cylinder to an inverted position. Return slowly to the horizontal position, again allowing the colloidal suspension to undulate slowly back and forth several times. Slowly return to the upright position. Repeat this entire procedure of mixing two additional times.

Allow colloidal suspension to stand at room temperature for 20 minutes. It

is now ready for use in the reaction. Note: the maximum length of time that the colloidal suspension remains active has not been determined.

Ascertaining the Optimum Serum Dilution

Two *hemolytic units* of colloidal suspension are used in ascertaining the optimum dilution of the serum; that is, the dilution of the serum at which a known negative serum does not inhibit the hemolytic power of the lecithin, while on the other hand, a known positive serum will completely inhibit hemolysis. To ascertain this optimum dilution a series of serum dilutions in arithmetic progression is set up and tested until the proper dilution is found. For example, if it is found that a 1:14 dilution of the known serum controls will fulfill the above requirements, this dilution is then employed in the actual tests when this "antigen" is used (Table I).

TABLE I

Showing the optimal serum dilution—in this case 1:14

	DILUTIONS					
	1:12	1:13	1:14	1:15	1:16	1:17
Positive serum	+	+	+	−	−	−
Negative serum	+	+	−	−	−	−

Preparation of Erythrocyte Suspension (4)

A 2% suspension of washed sheep cells in 0.85% saline is prepared according to the method of Kolmer. Defibrinated cells 48 hours old were employed.

Performance of the Test

1. Pipette into a Wassermann tube 0.4 ml. of the patient's diluted serum (inactivated).
2. Add 0.4 ml. of the colloidal suspension of "antigen". Shake.
3. Add 1.7 ml. of 0.85% saline.
4. Place in a 37° C. water bath for 30 minutes.
5. Remove from bath and place in a refrigerator at 4° C. for 19 hours.
6. Remove from refrigerator and allow to stand at room temperature for 1 hour.
7. Add 0.5 ml. of a 2% sheep cell suspension.
8. Place in a 37° C. water bath for 5 minutes.
9. Remove from water bath and read the degree of hemolysis. If necessary, make a second reading after 4 to 5 hours at room temperature.

DISCUSSION

This preliminary report puts forward the concept of using a test material with hemolytic properties but which is rendered inactive by syphilitic antibodies in place of a non-hemolytic antigen plus complement and amboceptor. It must be emphasised that the reaction described is not as yet a practical serologic test for syphilis.

Since the test employs erythrocytes as an indicator, it may be described as belonging to the hemolytic group of serologic reactions; but, as it also involves a simple union of reagin with lipids, it may also be placed in the group of flocculation reactions. The mechanism of the reaction may be explained as follows: if the hemolysing material, i.e. lecithin, combines with the reagin in the patient's serum, the hemolysing material will then remain in a state of colloidal dispersion as a result of the protective action of the reagin; and, therefore will not be capable of causing hemolysis of the erythrocytes. If, on the other hand, there is no reagin present, the hemolysing lipid is then in direct contact with the erythrocytes resulting in hemolysis of the cells because of the disturbance of the physiologic pattern on the surface of the erythrocytes.

Theoretically and experimentally it has been shown that the reaction described is based upon the principles which underlie both the complement-fixation and flocculation tests; and, it may thus be considered a link between these two types of serologic reactions.

It is not yet certain that the optimal conditions for the performance of the test have been determined. The type of lecithin used also presents a problem. By chance a partially oxidized, old, egg-lecithin preparation was first used. This lecithin was found to possess a high hemolytic activity and its ether-soluble fraction showed a selective affinity toward the reagin of positive syphilitic serums. When another lecithin sample (Amend No. C 67330) was used, no water-soluble fraction was present and the hemolytic activity was almost nil. With additions of cardiolipin and cholesterol attempts are being made to increase the sensitivity of the hemolysing material.

The reaction is not yet sufficiently sensitive or specific. It was noted, however, that strongly positive syphilitic serums all give a positive reaction with the present technic described. It was further observed that following adequate antisyphilitic treatment the reaction with this test became negative earlier than did the Kahn and Wassermann reactions.

SUMMARY

A hemolytic reaction is described employing phospholipid material with hemolytic properties in which complement and amboceptor are not required.

The reaction is based upon the accepted principles underlying all hemolytic and flocculation tests.

The phospholipid used was an ether extract of a commercial, partially oxidized, aged egg-lecithin, which possessed both hemolytic activity and a greater affinity for positive syphilitic sera than for negative sera. These properties are not necessarily present in all samples of lecithin.

Addition of suitable quantities of cardiolipin and cholesterol increases the affinity of the hemolysing material toward positive sera without affecting its hemolytic activity.

It is not possible in this preliminary report to state whether the reaction described is sufficiently specific and sensitive, or that the optimal conditions for the reaction have been determined.

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